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PROCESS FOR OBTAINING HIGHLY-PURIFIED ALGINATES

5   The invention relates to a process for obtaining highly-purified
alginates, in particular from brown algae, and alginates
manufactured with this process with a high degree of
polymerisation, and the use thereof.

10   Alginates have a large number of applications in the food
technology sector (e.g. Askar in "Alimenta", Vol. 21, 1982, p.
165 ff.) and in textile technology, and increasingly in the
pharmaceutical sector, medicine, biochemistry, and
biotechnology. The alginates acquired according to the processes
15   known hitherto from algae plants (for an overview, see for
example D.J. McHugh: "Production and Utilization of Products
from Commercial Seaweeds" in "FAO Fisheries Technical Papers",
Vol. 288, 1987, Chap. 2) are characterised by fluctuations in
composition and structure, as well as by impurities. This
20   derives from the fact that the raw alginate is extracted from
biomass which is acquired from the wild population. The algae
populations which grow in coastal waters in particular are
subjected to numerous geographical, seasonal, and substance
influences (environmental pollution). Added to this is the fact
25   that, during the harvest, the algae may be collected together
with foreign substances, and are subjected to chemical treatment
for preservation and decolouration (e.g. with formalin and/or
hypochloride).

30   The raw alginates available hitherto are therefore mixed
polymers of variable structure with impurities, which may
include in particular toxic chemicals. In view of the fact that
in food and textile technology interest pertains in particular
in the formative properties of the alginates, processes are
35   being developed for the subsequent purification of the raw
alginates, as explained hereinafter, in the first instance for
biological-medical applications.

Processes for the purification of alginates (such as are available, for example, from the company Keltone LV or Kelco Nutrasweet) are described, for example, in DE-OS 4 204 012, US-A 5 429 821 (or US-A 5 656 468) and in the publication by P. De

5 Vos et al. in "Diabetologia" (Vol. 40, 1997, p. 262 ff.). These processes generally have the disadvantage of a high expenditure of energy (applications of electrophoresis, freeze-drying, or centrifuging, heating, or boiling), a high environmental burden (use of acids such as HCl, H_2SO_4, non-biodegradable solvents such

10 as $CHCl_3$, or heavy metal ions such as barium, lead, or cadmium), and a restriction on the attainable mean molecular weight of the purified final material. Further disadvantages of the conventional processes are derived individually from the following explanations:

15 With the process known from DE-OS 42 04 012, a raw alginate solution is subjected to treatment with a complex forming agent, acid extraction at high temperatures (about 70 °C), washing, treatment with concentrated alcohol (about 80 %), and further

20 treatment with a complex forming agent. A dialysis process and freeze drying follows (or electrophoresis or centrifuging) to obtain the purified alginate. This process is disadvantageous because of the high energy expenditure, the large number of process stages, the use of toxic materials (e.g. barium as the

25 complex forming agent) and the restriction to alginates (< 500 kD). One special problem, however, is that the purification effect of this process is only restricted.

In DE-OS 42 04 012 the purified alginates are indeed referred to

30 as nitrogen-free substances, but only on the assumption of freedom from mitogens. It has however been shown that the animal experiments developed in the 'eighties and implemented in DE-OS 42 04 012, are not suitable for demonstrating freedom from mitogens, such as would fulfil the requirements of modern

35 biomedical applications, such as implant technology. Thus, for example, the animal experiments with the conventional purification process were carried out with what are known as Lewis rats. In the interim, however, it has been proved by

G. Klöck et al. in "Biomaterials" (Vol. 18, 1997, p. 707 ff.) and by P. Gröhn (Dissertation at the University of Würzburg, 1998) that Lewis rats have relatively low sensitivity to nitrogenic substances. Implanted alginate capsules, which did not induce any inflammation reactions in Lewis rats after three weeks, led, for example with what are referred to as BB-OK rats ("Bio Breeding / Ottawa Karlsburg"), to inflammation reactions. It follows from this that the nitrogen-free substances known from DE-OS 42 04 012 cannot in fact be regarded as highly-purified, and are only capable of restricted use due to reduced biocompatibility in biomedical applications.

With the purification processes known from US-A 5,429,821 and US-A 5,656,468, an acid precipitation is likewise carried out. Again, high-temperature process stages are required, and, for the final acquisition of the alginates, centrifuging, electrophoresis, or freeze-drying. The alginates acquired with this process are restricted to molecular weights of less than 200 kD. Drying follows at 80 °C, during which drying artefacts may be incurred in the mixed polymer due to structural or substance changes.

Another problem arises from the restriction to relatively low molecular weights. Thus, for example, it is known from "Immobilized Enzymes" by J. Chibata (A Halsted Press Book, John Wiley & Sons, 1978) that the bio-toxicity of materials decreases with the increase of the molecular weight.

In the final analysis, the process referred to heretofore according to P. de Vos et al. is also characterised by numerous process stages, including acid precipitation, the use of toxic chemicals (chloroform), the use of highly-concentrated ethanol (about 70 %), and the use of centrifuging, electrophoresis, or freeze-drying.

A further disadvantage of all purification processes consists of their restriction to the purification of commercially available raw alginates. The processes are not applicable to fresh

material or harvested biomass. In addition to this, the processes are not practicable for large-scale technical application because of the elaborate process performance, the energy expenditure, and the use of toxic chemicals.

5   The objective on which the invention is based is of providing a process for the obtaining of highly-purified alginates with which the disadvantages of conventional purification processes are avoided and the manufacture of highly-purified alginates is

10   rendered possible, in particular in large-scale technical production. The objective of the invention is also to provide a new type of alginate, in particular with a higher molecular weight and increased viscosity than conventional alginates.

15   This objective is achieved by a process and an alginate composition with the features according to Patent Claim 1 and 16 respectively. Advantageous embodiments and applications of the invention are derived from the dependent claims.

20   The term "highly-purified alginate" is to be understood in this context to be an alginate composition which is capable of reproducible manufacture, which features predetermined molecular weight and/or viscosity parameters, and high sterility, purity, and biocompatibility, the latter feature relating, for example,

25   to the fact that alginates which are highly purified in accordance with the invention do not induce any, or negligibly low, foreign body reaction in auto-immune diabetic BB/OK rats, even after several weeks of implantation.

30   By contrast with the conventional purification processes, which are all adapted to commercially available raw alginates, but which represent blends or mixtures of different algae materials, with the inclusion of animal or other foreign materials, and therefore cannot in principle provide highly-purified alginate,

35   according to the invention alginate manufacture or acquisition is achieved, which derives predominantly from clean fresh algae material or dried algae material as the source material. Provision is made in particular for the algae material to be

treated initially in the presence of complex forming agents, whereby sedimentation of cell constituents and particles takes place with a binding agent in the form of a granulate or a comparable porous material. After a filtration process, a
5 precipitation stage takes place, for preference under simultaneous blowing in of a carrier gas, under the effect of which the precipitated alginate floats on the solution. This foaming (floating) is not mandatorily required. Precipitated alginate can also be separated from the solution by other means
10 (e.g. by a decanting process). The alginate acquired in this way can be drawn off from the surface of the solution, or dehydrated in air or by a filter press as a residue after decanting. Depending on the application, this purification process, or part steps of this purification process, are carried out once or
15 several times. After the last purification, subsequent washing and air drying takes place.

This procedure has the following advantages. During the acquisition of the alginate, toxic chemicals and high-temperature conditions can be entirely done without. The
20 individual process stages are based on inherently known and easily mastered techniques, the combination of which according to the invention is particularly advantageous with regard to process costs, material investment, and process speed. For the
25 first time, by contrast with all conventional attempts at purification, bio-compatibility is achieved of the alginate material acquired, so that the area of application of this material is substantially increased, especially in the fields of biology and medicine. The biocompatible product manufactured
30 according to the invention is based exclusively on alginate. The alginate is free of additives, such as, for example, immuno-suppressants or immune-stimulating substances or phenols or compounds similar to phenols, and can be used directly in this condition.

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The stages of the process can be carried out without any problems on a relatively small scale, e.g. at the place of the algae harvesting, or can also be carried out on a technically

large scale. By specific control of the precipitation reaction, impurities caused by fucoidan will be avoided, with the result that the purity of the alginate acquired improves in comparison with conventional alginates.

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The direct processing of alginate material provides a series of advantages. Firstly, disadvantages with conventional algae harvests, which are caused by decomposition and rotting at the place of harvesting, and by preservative treatment with

10 chemicals, can be avoided completely. Secondly, the harvested algae can be separated according to organs or tissue sections, before the algae acquisition is carried out. Because the different algae tissues differ due to a variety of conditions of the monomer mannuronic acid and guluronic acid, specific highly-

15 purified alginates can be manufactured with a specific mannuron-guluron composition. The same applies accordingly to the choice of specific tissue parts for the achievement of a specific molecular weight. It is possible for alginate compositions according to the invention to be acquired with a molecular

20 weight of more than 1,000 kD. In the final analysis, the process according to the invention can be achieved without elaborate centrifuge stages, as a result of which the practical implementation is further simplified. The specific acquisition of different types of high-purity alginate (e.g. alginate rich

25 in guluronate and mannuron) can be achieved by the specific acquisition of specific algae species (Laminarales, Ectocarpales, Fucales, and other algae species containing alginate), which differ in the chemical structure of the particular alginate concerned. With the organ-specific

30 selection, by contrast, phylloids, cauloids, and rhizoids are separated from the algae and processed separately. This differentiation can be further refined in that individual tissues are separated from the different organs, and the alginate is acquired separately from these tissues.

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The invention is particularly well-suited to the acquisition of alginate from fresh or dried algae material (in particular brown algae). It is however also possible, with the process according

to the invention, for commercially available raw alginates to be
purified or for the process according to the invention to be
combined with specific washing or separation stages (e.g.
centrifuging), which are known from the conventional
5   purification process.

Advantages of the invention are derived from the fact that the
high-purity alginate is acquired directly from the algae plants
under control and supervision of all the process stages. Toxic
10   chemicals (solvents in particular) are avoided, so that use for
pharmaceutical purposes is possible without further ado. The
foaming of the precipitated alginate represents a particularly
simple and low-energy separation process.

15   According to a preferred embodiment of the invention, the
following process stages are carried out initially on fresh
algae material or dried material, and then partially repeated on
highly-purified alginate, which was acquired from the first or
earlier process sequences. The algae material or commercial
20   alginate is designated hereinafter as "source material".

According to the invention, the source material is initially
extracted in the presence of complex forming agents, if
appropriate in a soda solution (see below, Example 1).
25   Subsequently, cell constituents and particles present in the
solution are brought to sedimentation by the addition of a
granulate and, if required, by the addition of ion exchangers
(such as, for example, amberlite), and the solution is then
filtered. The sedimentation can, as an alternative, be carried
30   out with the use of electrographite (e.g. in globular form). In
this situation, electrographite is introduced into the solution
and charged by means of a current flow between two electrodes
suspended in the solution. The charged electrographite globules
show an accumulation of impurities, which in this case comprise
35   the cell constituents and foreign particles concerned. The
sedimentation is used for preference in the purification of
commercial algae material, but can also be dispensed with
entirely, depending on the application (see below, Example 2).

The filter stage may comprise a multiple filtering process with pore sizes being reduced step by step, e.g. from 15 µm to 0.1 µm. From the filtered solution, the alginate is precipitated through a suitable precipitation medium. The precipitation is

5 carried out for preference with an alcohol (e.g. ethanol). An acid or another suitable precipitation agent can, however, also be used. The alcohol concentration is selected in the range between 10 % to 50 %, for preference in the range from 30 % to about 50 %. In this concentration range impurities due to

10 immunologically active polysaccharides such as fucoidan remain in the solution, and can therefore be separated from the alginate. In the event of higher alcohol concentrations being used, such as in the process according to De Vos et al., these undesirable polysaccharides cannot be separated. During the

15 precipitation, for preference, the solution flows through with a propellant gas (e.g. air). The precipitated alginate is driven upwards by the air which is blown in, and can be easily separated from the surface of the solution by a suitable device (e.g. net, sieve, or the like). The alginate collected can then

20 be dewatered with a filter press.

The process stages referred to are repeated, depending on the application, either in whole, in part, or in a partially modified form. After the last process sequence, the highly-

25 purified alginate is washed in ethanol and, if appropriate, then washed in water and air-dried at room temperature.

The purified alginate has, as a function of the source material, a ratio of the monomer mannuronic acid and guluronic acid in the

30 range from 0.1 - 9 (corresponding to 1 % to 90 % mannuronic acid) and a mean molecular weight from approx. 10 kD to more than 1000 kD. Alginate purified in this way, implanted in an autoimmune diabetic BB/OK rat, does not incur any foreign body reaction after an implantation period of 3 weeks, or only a

35 negligibly small reaction, as is explained in greater detail hereinafter.

The process described for the acquisition of alginate can be

modified with regard to the precipitation medium, the choice of the sedimentation medium, the choice of the inert propellant gas, the precipitation process, and/or the procedure with the collection of the precipitated alginates. Instead of the

5 diatomaceous earth used for the sedimentation, any other absorbent material can also be used, such as electrographite, granulate, cellulose, or porous recycling materials in powder or particle form. It is also possible to use stirring equipment coated with an absorbent material.

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The object of the invention is also a highly-purified alginate which consists of a mixed polymer of mannuronic acid and guluronic acid in a proportion of the range from 1 % to 90 %, in particular about 70 %, whereby the mean molecular weight is

15 greater than 1000 kD, but at least greater than 250 kD.

Alginates according to the invention feature characteristic substance properties, due to their extreme purity and gentle extraction technique with the process according to the

20 invention, which are not found with conventional alginates. These substance properties comprise both characteristic parameters, which are directly measurable as properties of the alginate (e.g. viscosity), as well as parameters which, as properties of the impurities removed according to the invention,

25 indicate the high purity of the alginate according to the invention due to their complete absence or their negligibly small presence (e.g. fluorescence properties of impurities, initiation of immunological reactions in animal experiments and in cell cultures, etc.).

30

An alginate according to the invention is characterised by a high viscosity. An aqueous solution of an alginate according to the invention with a 0.1 % concentration has a viscosity in the range from 10 to 15 mPa s. This represents a substantially

35 higher value in relation to the viscosity of conventional alginate solutions of the same concentration (about 1 to 5 mPa s). With 0.5 % solutions of the alginates according to the invention, a viscosity is derived of 280 mPa s. The viscosity of

the alginate solutions is determined with a ball viscosimeter (type AMV-200, Anton Paar KG, Fraz, Austria). The molecular weight is determined from the viscosity values by means of the process according to Huggins ("J. Am. Chem. Soc.", Vol. 64,

5   1942, p. 2716 ff.) and Krämer ("Ind. Eng. Chem.", Vol. 30, 1938, p. 1200 ff.). With the alginates according to the invention, mean molecular weights are derived which are larger than 250 kD.

Alginates according to the invention are free of phenols and

10   compounds similar to phenols, in particular of polyphenols, which are composed of phloroglucinol, and phenol-protein compounds. With an excitation wavelength of 366 nm, alginates according to the invention, apart from a solvent fluorescence (Raman bands of the water) at 418 nm in the spectral range from

15   380 to 550 nm, do not have any fluorescence emissions. The freedom from phenols and polyphenols of the alginates according to the invention is also derived from colour tests with the use of the Folin-Denis reagent or with dimethoxybenzaldehyde (DMBA). Alginates according to the invention do not have any extinction

20   during these colour tests, apart from the solvent media absorption.

Alginates according to the invention are practically free of substances (e.g. proteins) which absorb at a wavelength of 350

25   nm. This freedom from proteins is in turn manifested in a fluorescence measurement with an excitation wavelength of 270 nm, which, apart from the solvent medium fluorescence, does not emit any fluorescence in the range from 300 to 500 nm. The freedom from proteins of the alginates according to the

30   invention is also manifested with the photometric proof of protein according to Bradford ("Anal. Biochem.", Vol. 72, 1976, p. 248 ff.).

Alginates according to the invention are also endotoxin-free.

35   Endotoxins are impurities which, on implantation, may incur an immune reaction by the recipient, which also passes out of the cell walls of the bacterial concomitant flora of the alginates into conventional algae extracts. Alginate solutions according

to the invention (concentration: 0.25 %) have an endotoxin content of less than 14.5 endotoxin units per millilitre of alginate solution (measuring process: Quantitative endotoxin determination with Limulus amoebocytes Lysate test).

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Alginates according to the invention are biocompatible, by contrast with conventional alginate extracts, inasmuch as this is demonstrated by the XTT and MTT tests explained hereinafter and by experiments on BB/OK rats.

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Preferred uses of a highly-purified alginate of this nature are transplant surgery, in which living cells are enclosed in an alginate capsule, and are implanted in the body of a living being without the initiation of immunological reactions. The

15 highly-purified alginate can also be used in the food or textile technology sectors for increasing the compatibility of certain specific foodstuffs or substances. It is emphasised that the process according to the invention as explained heretofore is also well-suited for the manufacture of highly-purified alginate

20 with lower molecular weight, up to 1000 kD.

Further advantages and details of the invention are described hereinafter, in particular with reference to the appended figures. These show:

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Fig. 1   A sectional view of a nozzle head for the manufacture of alginate capsules;

Fig. 2   Fluorescence spectra for the demonstration of the
30   freedom from phenol of the alginates according to the invention;

Fig. 3   Fluorescence spectra for the demonstration of the freedom from protein of the alginates according to the
35   invention;

Fig. 4   Results of a lymphocyte simulation test for the

demonstration of freedom from immunogens of alginates according to the invention.

Examples of performance

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Hereinafter specific embodiments of the procedure explained heretofore are described, on the basis of examples. In this situation, reference is made without restriction to the purification or use of alginate material on the basis of brown

10 algae. Instead of brown algae, in general all salt-water or fresh-water algae containing alginate can be used. The purification of alginate material from other algae is effected in the corresponding manner.

15 The source material is comprised of (1) fresh brown algae, (2) dried brown algae, or (3) commercial alginate. As fresh material (1), brown algae harvested in nature or in a cultivation area or greenhouse, or brown algae portions from predetermined development stages of the life cycle of the algae or

20 corresponding algae material cultivated in a homogenous bioreactor are used. The drying material (2) consists of dry brown algae, acquired according to these alternatives.

Example 1

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In the first example, reference is made to 80 g dry and then rehydrated (watered) algae. With commercial alginate, of which the dry weight makes up only about 1 % of the fresh weight, correspondingly less dry weight is used. The dry material (e.g.

30 Laminarales, Fucales) according to (2) or (3) are watered for several hours in hot mains water, in accordance with the initial quantity. This may last, for example, for at least 3 to 4 hours with mains water with a temperature of 40 °C, whereby the water is replaced several times, or flows. With the use of cold water,

35 the watering must be extended accordingly. Watering is effected for preference by the material being suspended in a water-permeable container (e.g. water-permeable bag) in flowing mains water. With watering in standing water, the material is, for

example, watered in 3 to 6 litres of water at an initial dry weight of about 80 g of dry algae (corresponding to 10 % of the fresh weight of about 800 g). After watering, the procedure takes place in an analogous manner for all three types of source
5   material (1) to (3) mentioned.

The material is suspended in about 5.7 litres of a 23 mN EDTA solution (distilled or desalinated water) or, in the case of commercial alginate (3), is dissolved. The effect of the
10   ethylene diamine tetra acetic acid (EDTA) solution takes place for at least 10 hours. The effect time can be shortened if the suspension is constantly stirred. As the effect time increases, so the yield of purified alginate improves. With an unstirred suspension, for example, an effect time of several days can be
15   provided for.

5 % Na_2CO_3 and EDTA as solid substances are now added, under stirring. The EDTA quantity is selected in such a way that a 50 mN EDTA solution is formed. The suspension is stirred until a
20   homogenous (fine dispersed) solution is achieved. This state is achieved in particular if only a few cell constituents are still visible in the solution. Some 34 g diatomaceous earth is then added under stirring, and the solution is stirred for at least 2 days. It is possible, as an alternative, for the diatomaceous
25   earth to be added together with Na_2CO_3 and EDTA under stirring. Provision can be made, depending on the application (in particular as a function of the brown algae material used), for ion exchange material additionally to be added simultaneously with the diatomaceous earth or electrographite. It is possible,
30   for example, for 34.2 g amberlite to be added as an ion exchanger, which has been subjected to purification beforehand. This purification serves to remove toxic substances and comprises watering in flowing water (duration about 3 hours).

35   After the diatomaceous earth treatment, the volume of the solution is diluted to 22.8 l with desalinated water, in order to reduce the viscosity. The dilution is selected in general in such a way that the solution is subsequent capable of being

filtered. The addition of water accordingly depends in particular on the brown algae material used. After the dilution and brief stirring, the solution is allowed to stand for at least 10 hours, in order for solid constituents to sediment out.

5   The standing time may be of the order of days. The supernatant is decanted off and filtered. The filtration takes place in multiple stages, whereby initially a deep filter with a pore size of 15 μm and then a filter with a pore size of 0.1 μm is used.

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The addition of salt to the filtrate then follows. The addition of KCl is preferred, whereby, depending on the application, other appropriate salts may also be added. So much KCl is added as solid substance as will produce 0.13 M KCl solution.

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An ethanol precipitation follows. The quantity of ethanol added depends on how much fucoidan is present in the material. With the first ethanol precipitation, the ethanol concentration should not exceed 40 % in the presence of fucoidan, since

20   otherwise the fucoidan will precipitate in sympathy. In the example given, about 12 litres of 99 % ethanol is added, so that the final concentration of ethanol in the total solution amounts to about 34 %. Provision can also be made for the ethanol concentration to be selected as a function of the nature of the

25   precipitation of the alginate. By variation of the ethanol concentration, the result can be achieved that the alginate precipitates in thread form or in wad form. The attempt is made to achieve such a precipitation as far as possible, in order for the alginate to float and is capable of undergoing further

30   processing, as explained hereinafter.

Instead of ethanol, at least one other alcohol (e.g. isopropanol) or a precipitation acid can be added, whereby the concentration is based on the criteria indicated heretofore.

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During the precipitation process, a propellant gas flows through the solution (e.g. air). The precipitated alginate is driven upwards by the air being blown in, i.e. while it is being

formed, and can be easily lifted from the surface of the solution by a suitable device (e.g. net, sieve, or the like). The precipitated alginate can also be collected without the addition of propellant gas by decanting or stirring with a

5 stirring device, remaining adherent to the precipitated alginate. The collected alginate is then dewatered with a filter press, in order to remove the water at least in part from the strongly hygroscopic material.

10 The procedural stage carried out up to this point are repeated, depending on the application, in full, in part, or in part under modified conditions. Further processing of the precipitated alginate is then carried out as follows.

15 The precipitated alginate is dissolved in 11.4 l and 0.5 M-KCl/10 mM-EDTA solution. Dissolving takes place under stirring, until a homogenous solution is achieved. A second precipitation then takes place with about 10 litres of a 99 % ethanol solution under the introduction of propellant gas. Under these conditions

20 the alcohol concentration in the total solution amounts to about 44 %. For this second precipitation, a higher alcohol (or acid) concentration can be selected, since the fucoidan (see above) is already separated. The concentration of the precipitation medium is again adjusted in such a way, however, that the formation of

25 wad-type alginate is promoted. With an alcohol concentration which is not optimum, the alginate is of the nature of gelatine and therefore cannot float (floating in the surface under the effect of propellant gas).

30 The precipitated alginate is then again dewatered through a filter press and washed several times with ten times the quantity of a 70 % ethanol solution. The material is then dried at room temperature. The drying can take place under sterile conditions. After the washing with ethanol, provision can be

35 made for the material to be washed several times with desalinated water or dialysed against desalinated water, in order to remove residual traces of concomitant substances.

The number of precipitations carried out according to the invention is based on the impurities or the toxic byproducts in the source material.

5 The tissue compatibility of the alginate acquired according to the Example is tested as follows. Implantation experiments are carried out with normoglycaemic (6.1 + 0.4 mM plasma glucose), diabetes-prone BB/OK rates (200 + 25 days old). These rats have a substantially higher sensitivity to impurities in alginates
10 than the Lewis rats referred to earlier.

Ba^{2+} alginate capsules were manufactured from the highly-purified alginate in accordance with the process described in DE-OS 42 04 012 A1. The alginate capsules have a mean diameter of 200 µm to
15 400 µm. Implantation was effected beneath the kidney capsule of the BB/OK rats. The recipient animals remained normoglycaemic and showed no loss in body weight. The animals were killed after three weeks, the kidneys removed, and fixed in Bouin's solution. After embedding in paraffin, the preparation of 7 µm sections
20 was carried out. Every 20th section from two independent individuals were taken for histological examination.

The result of the investigation for different samples in comparison with raw alginate in compiled in the following table:
25 Result for two independent samples:

Sample	Rat #	Histological evaluation	Endotoxin content of a 0.25 % solution of the alginate
S1	V159	(+)	1 EU/ml
30	V161	+	
S2	V454	0	3.5 EU/ml
Raw alginate	V12	++++	> 1000 EU/ml

(Legend:
35 0: No reaction, (+): very weak reaction, +: weak reaction, ++++ very strong fibrosis)

It can be seen that the implantation with the alginate according

to the invention does not cause any reaction, or only a very weak one, while by contrast implantation with commercially available raw alginate incurs a very strong fibrosis.

5   The endotoxin content, which is characteristic of a potential incidence of bacteria, shows excellent values in the case of highly-purified alginates, values which are virtually negligible, while by contrast the comparison value of commercial raw alginate is about a thousand times greater.

10

It was determined with a highly-purified alginate according to the invention, with the use of the following test procedures, that no impurities of toxic substances were incurred. The test procedures comprise in particular fluorescence spectroscopic

15   processes and endotoxin or mitogen activity essays, such as are described in G. Klock et al. in "Appl. Microbio. Biotechnology" (Vol. 40, 1994, page 638 ff.) and in "Biomaterials" (Vol. 18, 1997, page 707), NMR-spectroscopic processes, the determination of the antioxidative potential of the highly-purified alginate

20   by determination of the reaction to the addition of HOCl, and by the determination of the oxidative activity of neutrophilic granolycytene, with the aid of chemiluminescence (see K. Arnold in "Proceedings of the Saxony Academy of Sciences in Leipzig", Vol. 58, 1997, Book 5) and the process of "Free Flow

25   Electrophoresis", as described by U. Zimmermann et al. in "Electrophoresis" (Vol. 13, 1992, page 269). No toxic contamination was determined with this process.

According to the process described in the publication referred

30   to above by G. Klöck et al. (1997), in addition, the proportion of mannuronic to guluronic acid was determined with the aid of what is referred to as "Circular Dichroismus-Spectroscopy" or with IR spectroscopy. In addition, the molecular weight was determined by the analysis of the viscosity.

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The capsules of alginate cross-linked with Ba^{2+} possess excellent elasticity, as was proved with the aid of compression measurements.

Example 2

With the second example, 10 g dry algae was taken as the
reference. With larger initial quantities, the quantitative
sizes given hereinafter are to be converted accordingly in
linear manner. As a departure from Example 1, the acquisition
and purification of alginate is effected to advantage without
watering. The dry source material is, instead, added directly
into the EDTA solution. The EDTA solution has a concentration in
the range from about 10 to 50 mM. The suspension is stirred for
24 hours and then screened to remove solid material. A further
advantage of the process explained according to the second
Example is that the EDTA consumption, and also the use of
alcohol, is reduced.

Provision can be made for additional activated carbon to be
added to the suspension during the EDTA treatment. The amount of
the activated carbon added amounts for preference to some 10 to
200 % of the dry algae mass which is weighed in.

This is followed immediately and without a separate
sedimentation stage by a filtration of the suspension. The
filtration takes place in two stages, whereby a pre-filter or
deep-filter is used first, with a pore size of 15 µm, and then a
filter with a pore size of 0.2 µm.

After the addition of salt to the filtrate as in Example 1 (e.g.
formation of a 0.13 M KCl solution) several ethanol
precipitations take place. For the first precipitation, 99 %
ethanol (denatured with acetone) is added, with the result that
a final concentration of the ethanol in the total solution of
37.5 % is attained. The precipitation product is collected and
added to a 0.5 M KCl solution (without EDTA). The volume of the
KVl solution is adjusted to a third of the solution volume prior
to the precipitation.

For the second precipitation, 99 % ethanol (denaturised with

acetone) is added, so that a final concentration of ethanol in the total solution of 44 % is attained in this case. The precipitation product is collected and added to bi-distilled water with a volume corresponding to the volume of the KCl
5 solution after the first precipitation.

Before the third precipitation a dialysis can be carried out of the precipitation product derived from the second precipitation. The dialysis, which is not a mandatory feature of the process,
10 is effected for a period of three days, with a change of water three times per day. For the third precipitation, 99 % ethanol (denaturised with acetone) is again added to adjust a final concentration of the ethanol in the total solution of 50 %. The precipitation product is collected and dried.
15

Example 3

An example for transplant surgery is described hereinafter, namely the micro-encapsulation of the Islets of Langerhans.
20

Isolated Islets of Langerhans were suspended in a solution of 0.9 % NaCl and 0.5 % of the purified alginate. This suspension was introduced in fine drop form through a spray nozzle 10 shown in Fig. 1. The nozzle has a movable inner hollow tube 20 (inner
25 nozzle) with a lur connection (inner diameter 350 µm or 2 mm), a middle hollow tube 30 (middle nozzle) with a diameter of 1 mm or 3.5 mm respectively and an outer channel 40, which opens into an adjustable air focusing head 50. These elements were fitted to a nozzle head 60, at which compressed air and lur connections are
30 located.

The Islet suspension in alginate is pressured through the central nozzle channel. A solution of 0.5 to 2 % of the alginate in a 0.9 % common salt solution is applied through the
35 surrounding channel. With the drops which occur at the nozzle outlet in this way, the outer alginate solution (0.5 to 2 %) surrounds the islets suspended in the 0.5 % alginate solution. Compressed air is introduced through the outer, third channel,

which shears the drops off from the nozzle aperture. The compressed air was adjusted to 30 to 40 mbar (7 to 8 1/min). The alginate drops 70 were delivered in 40 mL cross-linking solution with 20 mM BaCl2 and 5 mM histidine. The cross-linking solution

5   is adjusted with NaCl to a physiological concentration (290 mOsmol). The capsules were then washed accordingly three times with isotonic common salt solution.

With the alginate according to the invention, general

10   encapsulations can be created for allogenic and xenogenic tissues, and for endocrinic tissues in particular.

Further characterisation of the alginate material according to the invention

15

1. Fluorometric test of phenol

Alginates according to the invention do not contain any contaminating impurities based on phenols, polyphenols, or other

20   phenol compounds. This freedom from phenols means that the impurities referred to are not contained in the alginates, or in such a small volume that applications in biology and medicine, and the applications referred to heretofore in particular, will not be interfered with by immune reactions or the like. The

25   freedom from phenol is demonstrated with a fluorometric analysis, as described by G. Skajk-Braek et al. (see "Biotechnology and Bioengineering", Vol. 33, 1989, p. 90 ff.). The measurement of the fluorescence is carried out with an LS50 spectrometer (Perkin-Elmer, Beaconsfield). At an excitation

30   wavelength of 366 nm, the fluorescence spectra in solution samples, as shown in Fig. 2, are derived during the purification according to Example 2. Before and after the initial filtration, the alginate solution shows a strong fluorescence in the range between 380 and 550 nm (upper spectra). After the filtration and

35   precipitation stages, the fluorescence is substantially decreased. The concentration of the final solution amounts to about 0.2 - 0.3 %. There remains only a fluorescence maximum at 418 nm, which corresponds to the solvent medium fluorescence. In

the alginate purified according to the invention, the fluorescence of the phenols and phenol compounds is reduced to less than about 10 % in relation to the unpurified alginate solution.

5

Fig. 2 shows that the phenol contents decrease drastically in the course of the process according to the invention, and that the purified alginate shows an emission which is only slightly above the values of highly-purified water.

10

2. Fluorometric test of protein

Freedom from protein of the alginate according to the invention is demonstrated as with the fluorometric analysis according to

15 1. Fig. 3 shows that, with an excitation wavelength of 270 nm before purification, a strong emission is measured in the range from 300 to 500 nm. This emission falls in the course of the purification to a value below 20 % of the fluorescence of the unpurified alginate solution. After the last precipitation,

20 fluorescence is no longer demonstrable, or is negligibly small.

3. Test of phenol according to Folin-Denis or with DMBA

The Folin-Denis proof colours all compounds which contain

25 phenol. Alginates manufactured according to the invention do not show extinction under this test. The Folin-Denis test is carried out under the following conditions:

The alginate solution which is to be analyzed (0.5 ml) is mixed

30 with 1 ml methanol for extraction. The specimen is placed in the refrigerator after intensive shaking for about 8 hours. The precipitated alginate is centrifuged off, and 10 µl of the residue is added to 40 µl of the Folin-Denis reagent (Fluka, Deisenhofen, Germany), 80 µl 1 M Na_2CO_3 and 120 µl H_2O (sequence

35 maintained). After intensive shaking, the colour development takes place in the water bath at 50 °C for 30 minutes. Measurement of the optical density follows with the aid of a photometer at a wavelength of 650 nm (or 725 nm).

With the addition of dimethoxybenzaldehyde (DMBA) too, the alginates according to the invention do not show any photometrically assessable colour reaction. This test was

5 conducted under the following conditions: The alginate solution to be analyzed (0.5 ml) is mixed with 1 ml methanol for the extraction. The sample is placed in the refrigerator after intensive shaking for about 8 hours. The precipitated alginate is centrifuged off. This is followed by the addition of 10 µl of

10 the residue into the DMBA solution, which is manufactured as follows: 2 g DMBA (Fluka, Deisenhofen, Germany) is dissolved in 100 ml glacial acetic acid. 16 ml HCl (37 %) is topped up to 100 ml with glacial acetic acid. Both solutions are to be mixed shortly before use, 1:1 to form the working reagent.

15

Measurement of the optical density then follows with the aid of a photometer at a wavelength of 490 nm (or 510 nm) against a calibration series of phloroglucinol (Sigma, Deisenhofen, Germany).

20

4. Protein test according to Bradford

The proof of protein according to Bradford (see "Anal. Biochem.", Vol. 72, 1976, p. 248 ff.) is based on the fact that

25 the colouring agent Coomassie brilliant blue bonds very specifically to proteins, as a result of which a colour break from red to blue takes place. The intensity of the blue colouring is in linear correlation with the amount of the protein, and can be quantified photometrically. With this

30 method, proteins can be demonstrated into the lower µg range. 10 µl of a 0.5 % alginate solution was examined. The proof substance containing the colouring agent added to the alginate solution was what is known as the Bradford Reagent (manufacturer: Sigma, Steinheim, Germany). After an incubation

35 period of 10 minutes, the sample absorption was measured photometrically at 570 nm, with the use of a "Thermomex Microplate Reader" device (manufacturer: Molecular Devices, Manlow Park, USA). No proteins were found in the alginate

solution according to the invention with this process.

5. XTT and MTT tests

5   The biocompatibility of the alginates purified according to the
    invention can be demonstrated with the following tests. Live
    cells, such as mouse lymphocytes or fibroblasts are kept in
    culture with the alginates for a few days. If the alginates
    demonstrate an immunogen effect, the cells will be stimulated or
10 inhibited (in order to make the effect of the stimulation
    clearer, additional stimulants, such as LPS or PHA, are added to
    the culture). The metabolic activity, increased with lymphocytes
    and decreased with fibroblasts, can be visualised on the basis
    of the conversion of a colouring agent (XTT or MTT) by
15 mitochondrial dehydrogenases and quantified photometrically.
    With alginates purified according to the invention, the
    colouring agent conversion is negligibly small (see Fig. 4),
    while with commercial alginates, by contrast, a sharp colouring
    agent conversion can be demonstrated. Fig. 4 shows the results
20 of the colour test for various different LPS pre-stimulations.

    The cultivation of the cells was carried out under the following
    conditions. The lymphocyte cell suspension ($1*10^6$ cells/ml in
    Complete Growth Medium) is mixed with various different
25 concentrations of lipopolysaccharides (LPS, final concentration:
    0.01 µg/ml) and alginate solution (final concentration: 0.05 %),
    and cultivated for three days at 5 % CO2 and 37 ºC. Thereafter
    the suspension is incubated with a solution of tetrazolium salt
    (XTT or MTT), and for a further 6 hours. This is followed by the
30 measurement of the optical density at 450 nm (reference
    measurement at 650 nm).

    6. Experiments in BB/OK rats

35 As described heretofore for Example 1, the biocompatibility of
    alginates according to the invention can also be demonstrated by
    in-vivo tests on BB/OK rats, the immune system of which features
    an increased macrophage activity. Alginate purified according to

the invention, implanted in accordance with the steps described in capsule form in rats' kidneys, did not cause any fibrosis after an incubation period of three weeks.

5   7. Electrorotation

The biocompatibility of the alginates purified according to the invention can also be demonstrated with the following test. Live cells, such as human lymphocytes or mouse lymphocytes, are kept
10   in culture with alginates for some days. If the alginates shown an immunogen effect, the cells react with a substantially increased membrane surface (increase in the microvilli; to make this effect clear, additional stimulants such as LPS or PHA may be added to the culture). Such an increase can be determined by
15   means of an increase in the specific membrane capacity C_m. The electrorotation of the cells under the effect of high-frequency electric fields offers a possibility of demonstrating these changes in the lymphocyte membrane on the level of the individual cell. With alginates purified according to the
20   invention, no increase in the specific membrane capacity can be measured, while by contrast with commercial alginates an increase in this value can be demonstrated.

The measurements were carried out under the following
25   conditions. The lymphocyte cell suspension ($1*10^6$ cells/ml in Complete Growth Medium) is mixed with an alginate solution (final concentration: 0.05 %) and cultivated for three days at 5 % CO_2 and 37 °C. Thereafter the suspension is washed twice in isoosmolar inositol solution (290 mOsm/kg), then the suspension
30   is thoroughly repeated. With different conductivity values (10, 25, 40 µS/cm), adjusted with HEPES-KOH, pH 7.2) the determination of the characteristic frequency (maximum) of the antifield rotation is carried out by the compensation method. With the aid of a linear regression of the characteristic
35   frequencies, normed with the cell radius, applied against the external conductivity, the electrical parameters (capacitance and conductivity) of the plasma membrane can be determined. The electrical parameters of the cells cultivated in the presence of

the alginates according to the invention remain unchanged, while by contrast the cells cultivated with commercial alginates feature an increase in membrane capacity by 10 to 50 %.

5   8. Cell size and cell number

The biocompatibility of the alginates purified according to the invention can also be demonstrated with the following test. Live cells, such as human lymphocytes or mouse lymphocytes, are kept

10   in culture with alginates for some days. If the alginates feature an immunogenic effect, the cells will increase in size and the number of cells will increase likewise (in order to make this effect clear, additional stimulants, such as LPS or PHA, can be added to the culture). The cell size and the number of

15   cells can be determined with the aid of the CASY1 Cell Analyzer (model TTC, Schärfe Technology, Reutlingen, Germany), which works on the Coulter principle. Such a device allows for the rapid and precise analysis (size, size distribution, and cell number) of several thousand cells per measurement. With

20   alginates purified according to the invention, no significant cell enlargement or increase in cell numbers can be measured after incubation, while with commercial alginates, by contrast, a clear cell enlargement (about 50 %) and an increase in the number of cells (35 to 50 %) can be demonstrated.

25

The measurements are carried out under the following conditions. The lymphocyte suspension ($1*10^6$ cells/ml in Complete Growth Medium) is mixed with an alginate solution (final concentration: 0.05 %) and cultivated for three days at 5 % CO_2 and 37 °C.

30   Thereafter an aliquot (100 µl), centrifuged off and absorbed in inosit (or PBS) of the lymphocyte suspension, after thorough repeat suspension, is washed twice in isoosmolar inositol solution (290 mOsm/kg), and absorbed in 10 ml PBS (phosphate-buffered saline) and measured immediately in the CASY1. From the

35   CASY1 histogram, the proportion of the coarse, stimulated cells in the total population can be calculated.